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A Simple, Rapid Extraction and Assay Procedure for NAD+-dependent Sorbitol Dehydrogenase (SDH) in Peach

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ABSTRACT. Sorbitol is the major photosynthetic product in peach [*Prunus persica* (L.) Batsch.]. In sink tissues, sorbitol is converted to fructose via NAD+-dependent SDH. A new procedure is described that allows rapid, simple quantification of SDH activity in growing tissues. The procedure uses only 0.01 to 5 g of fresh tissue per sample, such that a single shoot tip, a single root tip, or ≈5 g of fruit flesh can be assayed for SDH activity. Storage of samples at 4 or -20 °C overnight resulted in significant loss of enzyme activity. Thus, freshly harvested tissues were ground with sand in buffer at 2 °C in a mortar and pestle, and the homogenate was centrifuged at 3000 g_n to remove particulate matter and sand. The supernatant was desalted on a Sephadex G-25 column, and the eluent was assayed for SDH activity immediately. Activity was determined by measuring the production of NADH per minute in the assay mixture using a spectrophotometer (340 nm). Tris buffer at pH 9.0 was the best for extraction of peach SDH. Activity of SDH was strongly inhibited by dithiothreitol (DTT) in the extraction mixture and by DTT, L-cysteine, or SDI-158 in the assay mixture, similar to results reported for SDH from mammalian tissues. Peach SDH has a K_m of 37.7 mM for sorbitol and a pH optimum of 9.5, similar to those reported for apple (*Malus ×domestica* Borkh.) SDH. Unlike older protocols for SDH activity in plant tissues, the new procedure features reduced sample size (1110 to 1/100 of that which was previously used), smaller volumes of buffer, fewer buffer ingredients, greatly reduced time for sample preparation, yet comparable or higher values of SDH specific activity. Following the same procedure, SDH activity was also measured in *Prunus fremontii* Wats., *Prunus ilicifolia* (Nutt.) Walp., and Marianna 2624 plum (*P. cerasifera* Ehrh. *x P. munsoniana* Wight & Hedr.).

Sorbitol is a sugar alcohol found mainly in the Rosaceae where it occurs in all genera of the tribes Spiraeoideae, Pomoideae, and Prunoideae (Plouvier, 1963), and it is common in many fruit (Whiting, 1970). Little is known about sorbitol metabolism in plants even though it is the major product of photosynthesis in Rosaceous tree fruit (Bieleski, 1982). Webb and Burley (1962) reported a 3 sorbitol : 1 sucrose ratio in the bark of 'Golden Delicious' apple, and sorbitol was the principal transport material in loquat (*Eriobotrya japonica* Lindl.), peach, apple, Asian pear (*Pyrus serotina* L.), and French prune (*Prunus domestica* L.) (Hansen and Ryugo, 1979; Nii et al., 1994). Studies of breakdown of [^{14}C]sorbitol in fruit and other sink tissues indicate that sorbitol is converted primarily to fructose (Hansen, 1970) via SDH: D-sorbitol + $NAD^+ \leftrightarrow$ b-fructose + NADH + H^+.

Negm and Loescher (1979) first detected NAD+-dependent SDH activity in apple callus tissue. More recently NAD+-dependent SDH activity has been measured (Yamakiand Ishikawa, 1986) and related to sink-source interconversions in apple leaves and fruit (Loescher et al., 1982). The enzyme was purified for the first time from a plant source (apple fruit) by Yamaguchi et al. (1994).

In some species, the activities of sucrose metabolizing enzymes, such as sucrose synthase and acid invertase, are correlated with growth rate and provide a measure of sink strength or the ability of a growing organ to compete forphotosynthate (Sun et al., 1992; Sung et al., 1993, 1994). Also, it has been shown that the activity of some enzymes of the sucrose synthase pathway—identified as the dominant sucrose metabolic activity in sucrose sink tissues (Xu et al., 1989b)—can adapt to environmental stresses as well as to seasonal growth changes more sharply than others, arising the concept of adaptive versus maintenance enzymes in different species, including trees (Black et al., 1987; Sung et al., 1989a, 1989b, 1993; Xu et al., 1989a).

Similarly, the enzyme mannitol dehydrogenase regulates sink carbon use in celery (*Apium gruveolens* L.), a plant that produces and translocates mannitol in favor of sucrose (Williamson et al., 1995). Mannitol dehydrogenase may play a role in salt stress tolerance and disease resistance, as it regulates the mannitol pool size in active sinks, which may cause tolerance by osmoprotection or function as scavenger of active oxygen species commonly formed in presence of high stress or pathogen invasion (Stoop et al., 1996).

Based on these recent studies on sorbitol, mannitol, and sucrose metabolizing enzymes, it seemed reasonable that the activity of NAD+-dependent SDH could play a key role in determining competition for carbohydrates among developing organs and that it could be used as a measure of potential growth or sink strength in peach.

Current methods for extracting and assaying SDH are slow, cumbersome, and require large amounts of tissue, chemicals, labor, and time. Therefore, the objective of this study was to develop a simple, rapid SDH extraction and assay procedure for eventual use as a research tool to study sink strength in peach and other species that produce and translocate sorbitol.

Materials and Methods

PLANT MATERIALS. SDH was extracted from various growing tissues of different species belonging to the genus *Prunus* (Table

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Table 1. SDH activity (fresh mass basis) in various organs of different species.

Species	Cultivar	Organ	Tissue amount (g)	Activity ±SE (nmol·min^{-1}·g^{-1})
Prunus persica	Encore	Shoot tip	1	243.3 ± 23.9
		Fruit	5	2.1 ± 0.5
	Flordaguard	Shoot tip	0.025	149.9 ± 12.5
		Root tip	0.015	200.6 ± 93.1
	Nemaguard	Shoot tip	0.5	149.9 ± 19.9
		Shoot tip	0.05	311.3 ± 14.7
		Terminal internode	0.035	90.7 ± 10.9
		Root tip	0.35	244.3 ± 8.0
	Nemared	Shoot tip	1	207.4 ± 9.2
		Root tip	1	164.1 ± 13.3
Prunus ilicifolia		Shoot tip	2	10.2
Prunus fremontii		Shoot tip	1	15.5
Prunus cerasifera X ***P. munsoniana***	Marianna 2624	shoot tip	0.35	100.3 ± 14.5

1). Shoot tips consisted of the apical meristem and all the folded leaves considered as mostly importing organs or active sinks. Their length was ≈1.5 to 3 cm and their fresh mass was ≈50 to 100 mg. Root tips consisted of the apical meristem and the following 3 cm of mainly elongating tissue and the individual fresh mass ranged from 10 to 20 mg. SDH was measured in 50 to 120 g 'Encore' peaches, but only after the pit hardening phase of growth. Samples were harvested all at once (usually in the morning) to reduce variation due to fluctuation in the daily metabolism of plants and were quickly transported from the greenhouse or field to the lab in aluminum foil on ice.

ENZYME EXTRACTION AND ASSAY PROCEDURE. SDH was extracted by homogenizing tissues in 0.2 M K-phosphate buffer (pH 9 at 25 °C) containing 8% (v/v) glycerol (buffer A) or in 0.1 M tris-HCl buffer (pH 9 at 25 °C) and 8% (v/v) glycerol (buffer B); 0.1% (v/v) Tween 20 and 1% (w/v) PVPP were added during grinding. When used, 2-mercaptoethanol was added to the buffer immediately before enzyme extraction since storage in buffer resulted in significant loss of SDH activity. The tissue was ground in buffer and sand using a precooled (2 to 4 °C) mortar and pestle. The homogenate was filtered through a layer of miracloth and centrifuged at 3000 g_n for 15 min. The supematant was desalted at 4 °C using a Sephadex G-25 (medium) column (96% protein yield) to eliminate small molecules such as sugars and nucleotides (Fig. 1). In cases where the amount of tissue available for the extraction was extremely reduced (<0.09 g of fresh tissue), homogenates were centrifuged but not desalted.

The 1-mL final volume assay mixture consisted of 0.1 mL desalted extract, 0.1 M tris-HCl buffer (pH 9.5 at 25 °C) or 0.2 M K-phosphate buffer (pH 9.5 at 25 °C), 1 mM NAD^+, and 300 mM sorbitol. The assay mixture minus sorbitol was incubated at 25 °C for 5 min and the reaction was started by adding sorbitol. Enzyme activity was determined by reading the change in absorbance per minute at 340 nm in a Spectronic 21-D (Milton Roy, Rochester, N.Y.) over a 5-min period (Fig. 1). The net change in absorbance per min (total change − background reaction) was then multiplied by the reaction volume (mL) and divided by the millimolar absorptivity of NADH at 340 nm (0.00616 for the Spectronic 21) times the extract volume (mL) to determine the amount of NAD^+ reduced to NADH.

Protein content was determined by the method of Bradford (Bradford, 1976). SDH specific activity was expressed as nanomoles of NADH produced per minute per milligram of protein or as nanomoles of NADH produced per minute per gram of fresh mass.

The pH optimum and K_m value for the enzyme were also determined to establish similarity with the enzyme extracted from apple and mammalian tissues. For determining the K_m value, $(NH_4)_2SO_4$ precipitation was used. After a first centrifugation of the crude extract at 24000 g_n for 15 min, the supematant was brought to 20% saturation with $(NH_4)_2SO_4$, centrifuged again at 24,000 g_n for 15 min, and the pellet was discarded. The supematant was then brought to 40% saturation by further addition of solid

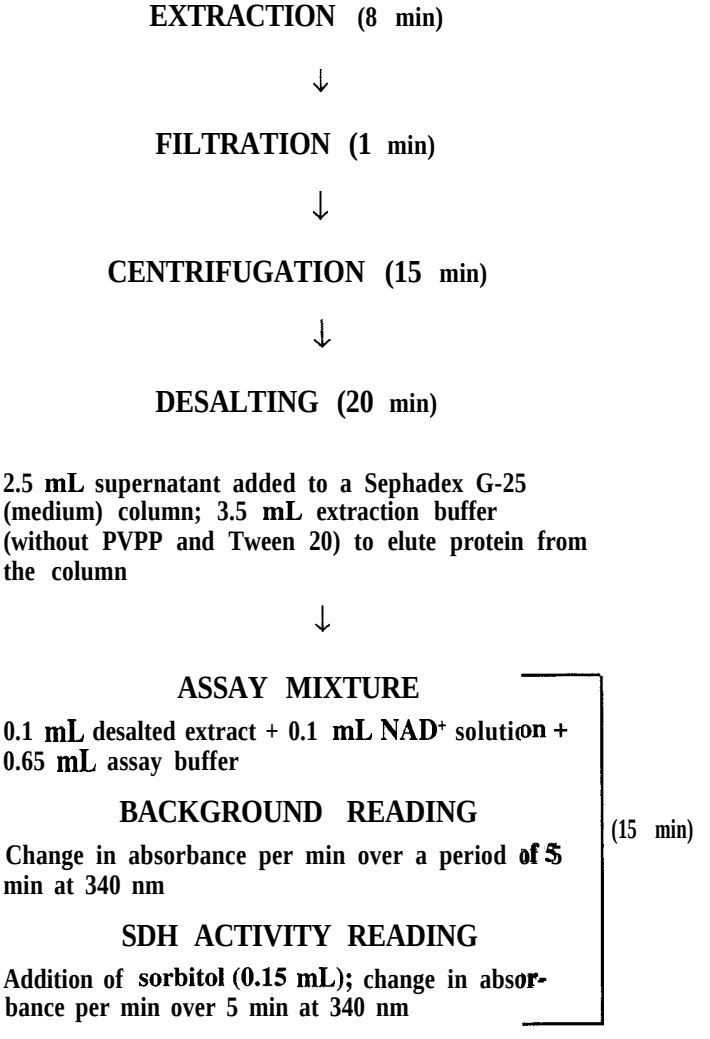

Fig. 1. Procedure of the assay for SDH and time required at each step.

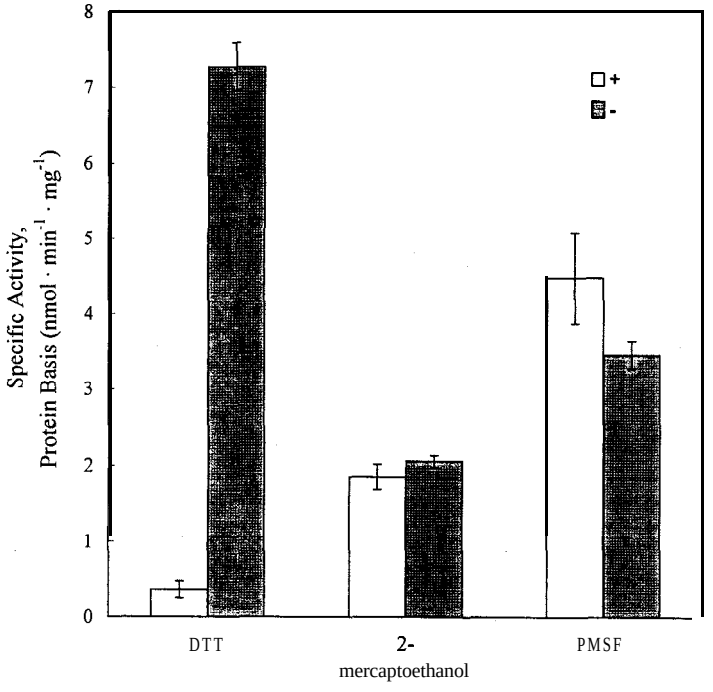

Fig. 2. SDH specific activities with (+) or without (-) the addition of dithiothreitol (DTT), 2-mercaptoethanol, or phenylmethylsulfonyl fluoride (PMSF) to the extractionmixture. Data for each pair of bars are from separate experiments. Error bars represent standard errors of the mean. Differences between + and – for PMSF and 2-mercaptoethanol were nonsignificant at $P \leq 0.05$.

$(NH_4)_2SO_4$, centrifuged, and the pellet was retained for determination of enzyme activity.

Enzyme stability and inhibition. Preliminary tests indicated that SDH activity decreased during and soon after extraction. To minimize this effect, three experiments were conducted using dithiothreitol (DTT), 2-mercaptoethanol, and phenylmethylsulfonyl fluoride (PMSF), which reduce oxidation and degradation by proteolytic enzymes (Scopes, 1994). The buffers used for extraction were buffer B (control), buffer B plus 2 mM DTT, buffer B plus 10 mM 2-mercaptoethanol, and buffer B plus 5 mM PMSF. The enzyme activity was measured as described above.

Stability of SDH over time was also tested in stored samples. Samples were collected and SDH activity was measured on the same day and after storing them for 24 hat 4 or -20 "C. The activity was measured as described above using buffer B for extraction.

A few compounds—$AgNO_3$, L-cysteine, DTT, and SDI-158 (a specific inhibitor of mammalian SDH; Geisen et al., 1994)—were included in the assay mixture at different concentrations to see whether SDH activity could be inhibited in vitro.

SDH in various sinks of different species. To verify the ease and reliability of our extraction and assay procedure, SDH was extracted from shoot tips, terminal internodes, fruit, and root tips of different peach cultivars and other *Prunus* species (Table 1). The assay was also conducted with extremely reduced amounts of tissue to see whether SDH activity could be accurately measured on single, tiny sink organs such as a single shoot tip or even a single root tip.

Paired *t* test and standard errors were used to analyze data of all the experiments described above.

Results and Discussion

A preliminary study showed that there was SDH activity loss when tissues were homogenized in the presence of liquid N_2. Thus, all the extractions done in the present study were conducted with precooled mortar and pestle and without liquid N_2.

Grinding the sample in tris-HCl and glycerol (buffer B, pH 9) without DTT, 2-mercaptoethanol, or PMSF gave the best results in terms of maximum activity and ease of buffer preparation, as far as extraction is concerned. When shoot tips were stored at 4 or-20 °C for 24 h, ≈50% of SDH activity was lost. Therefore, enzyme must be extracted and assayed immediately after harvest of samples. Tris-HCl (0.1 M) at pH 9.5 was the best buffer for the assay mixture. This result agrees with previous work conducted on apple tissue (Negm and Loescher, 1979; Yamaguchi et al., 1994).

The K_m value obtained in this study for the oxidation of sorbitol (37.7 mM) is very similar to the one obtained by Yamaguchi et al. (1994) in apples (40.3 mM), confirming the similarity between the two enzymes.

A 14-fold decrease in SDH specific activity was detected when DTT was present in the extraction buffer (Fig. 2). On the other hand, samples extracted in buffer containing 2-mercaptoethanol or PMSF did not have significantly different activity from the control (Fig. 2).

$AgNO_3$, known as a potent inhibitor of many enzymatic systems, completely inhibited SDH activity at a concentration as low as 0.2 mM, while 27% of activity was still present when 10 mM L-cysteine was added to the assay mixture (Table 2). The inhibitory action of DTT in the assay mixture appeared to be weaker than that observed when DTT was included in the extraction buffer (Fig. 2, Table 2). This is probably due to the longer incubation time of enzyme and inhibitor when DTT is included in the extraction buffer. The specific inhibitor SDI-1.58 fully repressed SDH activity at a concentration of 5 mM. This suggests that some similarity exists between plant and mammalian SDH, since thiol compounds are potent inhibitors in the latter (Lindstad and McKinley-McKee, 1996).

Maximum SDH specific activities obtained here from peach shoot tips (9.5 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein or 3 11 $nmol \cdot min^{-1} \cdot g^{-1}$ fresh mass) were comparable to those obtained by Loescher et al. (1982) in apple leaves (3.05 to 7.95 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein) using ammonium sulfate precipitation and overnight dialysis. Also, Yamaki andIshikawa(1986) obtained a maximum activity of ≈0.12 $nmol \cdot min^{-1} \cdot g^{-1}$ fresh mass in apple leaves and 140 $nmol \cdot min^{-1} \cdot g^{-1}$ fresh mass in apples using ammonium sulfate precipitation, dialysis, and a DEAE-cellulose column. In a more recent work, Yamaguchi et al. (1994) purified SDH from apples and obtained a much higher specific activity (13 10 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein). However, a specific activity of 10 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein after the first two steps of purification (ammonium sulfate + butyl-toyopearl column and

Table 2. Relative SDH activity (% of control) when inhibitors are added to the assay mixture at different concentrations.

Compound added	Final concn (mM)	Relative activity (%)
Control	---	100
$AgNO_3$	0.2	0
Dithiothreitol	1	68
	2	54
	10	19
L-cysteine	1	89
	5	56
	10	27
SDI-158	1	32
	2	27
	5	0

DEAE-cellulose column; 1.4-fold purification) was comparable to our result using only a desalted crude extract. Merlo and Passera (199 1) were also able to determine SDH activity in crude extract of peach young leaves desalted with a Sephadex G-25 column. However, using a different extraction buffer at pH 7.5, they detected a maximum SDH activity much lower than that obtained in the present work (≈83.3 compared to our 311 $nmol \cdot min^{-1} \cdot g^{-1}$ fresh mass).

Biological interpretations of datadealing with measurements of in vitro enzyme activity are sometimes difficult to make due to high variability among samples and lack of adequate replication. Our extraction and assay protocol reduces significantly the time per sample and cost of materials compared to previously published methods(Loescheretal., 1982; Negm and Loescher, 1979; Yamaguchi et al., 1994; Yamaki and Ishikawa, 1986), allowing increased replication.

Another advantage revealed by this work is a reduction in sample size compared to that used in previous studies. For example, Loescher et al. (1982) used 10 g of apple leaves, and Yamaki and Ishikawa (1986) used 20 g of apple leaves and 20 to 100 g of fruit flesh. Following the same procedure, we were also able to extract SDH and conduct an assay on a single shoot tip (0.05 to 0.1 g), 5 g of fruit flesh, or even a single root tip (0.015 g) (Table 1). This is particularly important when tracking the growth and development of a single organ or when plant material is a limiting factor. Enzyme activity was detected also in growing tissues of *Prunus* species other than peach (Table 1), proving that our extraction and assay protocol represents an efficient tool for the study of sink metabolism in species that produce and translocate sorbitol.

In conclusion, the extraction procedure developed here greatly simplifies classic methods for the measurement of SDH activity, eliminating sophisticated and time consuming steps such as those involved in protein purification and greatly reducing the amount of tissue needed per sample. As a result, greater replication of experiments and more reliable statistical analysis become possible, suggesting that the procedure can be used as a research tool to improve our knowledge of the role of sorbitol in plants.

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